RIP-Cre Revisited, Evidence for Impairments of Pancreatic β-Cell Function*

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The Cre/loxP recombinase system for performing conditional gene targeting experiments has been very useful in exploring genetic pathways that control both the development and function of pancreatic β-cells. One particular line of transgenic mice (B6.Cg-Tg(Ins2-cre)25Mgn/J), commonly called RIP-Cre, in which expression of Cre recombinase is controlled by a short fragment of the rat insulin II gene promoter has been used in at least 21 studies on at least 17 genes. In most of these studies inactivation of the gene of interest was associated with either glucose intolerance or frank diabetes. Experimental evidence has been gradually emerging to suggest that RIP-Cre mice alone display glucose intolerance. In this study experiments from three laboratories demonstrate that RIP-Cre mice, in the absence of genes targeted by loxP sites, are glucose intolerant, possibly due to impaired insulin secretion. In addition, we review the use of RIP-Cre mice and discuss possible molecular underpinnings and ramifications of our findings.

Cre-mediated deletion of genomic DNA fragments bracketed by loxP sites has become a standard tool for determining the function of specific genes in mouse development, physiology and disease. In the mouse, the expression of Cre is most frequently accomplished through conventional or BAC-derived fusion genes, infection with Cre-expressing recombinant viruses, or through the insertion of Cre coding sequences into endogenous genes by gene targeting. By varying the regulatory control regions, Cre can be directed to specific cell types and lineages and the temporal aspects of expression can also be modulated. However, the method used to direct Cre to a specific cell type may lead to undesired effects and often involves specific compromises. For instance, although easily and widely performed, the generation of transgenic mice by the method of pronuclear microinjection does not control for site of integration, which can both modulate and extend the expression pattern of Cre. This is more often an issue with short promoter-driven transgenes, since they appear to be more prone to so-called position effects than are much larger BAC-derived transgenes. On the other

hand, specific transgenic lines may exhibit traits that are advantageous, such as high levels of expression.

It has long been assumed that the expression of Cre recombinase does not adversely affect the physiology of the host cell. However, there have been reports on the apparent toxic effects of Cre (1–4). Experiments in mouse embryonic fibroblasts have demonstrated effects on cell growth and DNA damage (2, 4). Although it is not difficult to test for the independent effect of Cre when performing a conditional knock-out experiment, mice carrying only the Cre transgene are not always used as controls, perhaps due to the widespread impression that the expression of Cre has minimal if any effects on the cell.

EXPERIMENTAL PROCEDURES

Experiments on the RIP-Cre mice were performed independently in three laboratories (laboratories of L. H., M. F. W., and M. R.).

In the Hennighausen laboratory, six RIP-Cre mice (three males and three females) and 10 control mice (five males and five females) in a pure C57BL/6 background were obtained from the Jackson Laboratory and were maintained on a 12-h light, 12-h dark cycle and fed water an open formula National Institutes of Health-specified diet for maintenance, growth, and reproduction (NIH-07 rodent diet). Glucose tolerance tests were performed by intraperitoneal injection of sterile-filtered D-glucose (2 g/kg of fasted body weight). Mice were food-deprived for 15 h prior to the injection. Blood glucose values were measured immediately before and 15, 30, 60, and 120 min after glucose injection using MediSense Precision Xtra (Abbott Laboratories, Bedford, MA). All procedures and studies were conducted in accordance with National Institutes of Health guidelines and approved by the NIDDK Animal Care and Use Committee.

In the White laboratory, mice were obtained by intercrossing RIP-Cre mice with wild type C57BL/6::129 mice to generate heterozygous offspring. Blood insulin levels were measured by rat insulin enzyme-linked immunosorbent assay (Crystal Chem Inc., Chicagi, IL) at the indicated time intervals after intraperitoneal injection of D-glucose (3 g/kg body weight). Glucose tolerance test were performed after intraperitoneal loading with D-glucose (2 g/kg body weight) on 2-month-old male RIP-Cre and wild type mice. All the data used in Fig. 2 were from mixed genetic background mice, and S.E. instead of S.D. was used for Y error. Both male and female C57BL/6::129 mice heterozygous for the RIP-Cre transgene displayed similarly impaired glucose tolerance. Identical results were obtained with mice housed at the Joslin Diabetes Center or at the Harvard School of Public Health.

In the Ristow laboratory, RIP-Cre founders (kindly derived from Dr. Kulkarni's colony) were reported to be 66% C57BL/6 initially and were backcrossed with C57BL/6 (Charles River, Munich, Germany) three times prior to breeding of the study group. Mice were housed in a

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TABLE 1

Studies that have used the RIP-Cre mice to inactivate genes targeted with loxP sites

N.D., not determined or the information was not available from the paper; N.T., not tested; JAX, Jackson Laboratory; MAM, Mark A. Magnuson; HE, Helena Edlund; RIP-Cre mice described in Ref. 13 were generated by Dr. Pedro Herrera.

Targeted gene	Glucose intolerance	Impaired insulin secretion	Cre control[a]	Source	Strain	Ref.
ARNT	Yes	Yes	No	MAM	N.D.	24
COUP-TFII	Yes	N.T.	No	MAM	129SvJ; C57BL/6	10
PKCλ	Yes	Yes	No	MAM	N.D.	28
IRS2	Yes	N.T.	Yes	JAX	C57BL/6	17
HNF-4a	Yes	Yes	No[b]	MAM	CD1	22
SSTR5	Yes	Yes	N.D.	HE	N.D.	8
c-met	Yes	Yes	No	JAX	C57BL/6J	26
IRS2	Yes	N.T.	Yes	MAM	129Sv; C57BL/6	7
IRS2	Yes	N.T.	No	JAX	129Sv; C57BL/6	18
HNF-1β	Yes	Yes	No	MAM	129Sv; C57BL/6; DBA2	15
MEN1	N.T.	N.T.	No	MAM	N.D.	20
GR	N.T.	N.T.	No	Ref. 13	C57BL/6; CBAJ	13
Stat3	Yes	Yes	No	MAM	C57BL/6	14
Stat3	Yes	N.T.	No	MAM	129Sv; C57BL/6	16
MEN1	N.T.	N.T.	Yes	MAM	C57BL/6; FVB; 129Sv	12
Frataxin	Yes	Yes	Yes	MAM	>92.5% C57BL/6	21
Ca$_v$1.2	Yes	Yes	No[b]	MAM	C57BL/6	23
PPARγ	Yes	N.T.	Yes	MAM	129Sv; FVB/N; C57BL/6	27
IGF1R	Yes	Yes	No	MAM	129/Sv; C57BL/6; DBA-2	25
Foxa2	N.T.	No	No[b]	MAM	CD1	11
Tfam	Yes	N.T.	No	MAM	Mixed	9
InsR	Yes	Yes	Yes	MAM	129Sv; C57BL/6; DBA-2	19

[a] RIP-Cre only (no floxed alleles).

[b] Refs. 11, 22, and 23 included RIP-Cre mice with one floxed allele as control.

standard barrier facility according Federation of European Laboratory Animal Science Associations (FELASA) regulations including a 12-h light, 12-h dark cycle. Mice were fed standard rodent chow (Altromin GmbH, Lage, Germany). Glucose tolerance tests were performed by intraperitoneal injection of sterile-filtered prewarmed D-glucose (2 g/kg of fasted body weight). A minimum of three animals per sex and genotype were tested. Mice were food-deprived for 16 h before injection. Blood was drawn by retro-orbital puncture at given time points. Serum glucose was immediately determined using MediSense Precision Plus Electrodes (Abbott GmbH & Co KG, Wiesbaden, Germany). Mouse serum insulin levels were determined by enzyme-linked immunosorbent assay for rat insulin using a mouse insulin standard (both from CrystalChem).

RESULTS

RIP-Cre Mice—The role of more than 20 genes in either the development or function of pancreatic β-cells has been studied through cell-specific deletion using Cre-expressing transgenic mice. In particular, transgenic mice have been used that express Cre recombinase under control of regulatory regions from either the rat insulin II gene or the mouse Pdx1 gene. The so-called RIP-Cre strain (5) of mice has been used successfully in at least 21 studies targeting at least 17 different genes. This strain carries a fusion gene in which the expression of Cre is determined by a 706-bp fragment of the rat insulin II promoter. This strain was initially selected due to the high level at which Cre is expressed, and the efficient manner in which recombination was achieved within more than 80% of the β-cells (6). In most settings robust Cre expression is considered advantageous, since the rate of recombination is thought to directly parallel the amount of Cre. Although this line of mice is also known to express Cre in the brain (21) and specifically in the hypothalamus (6, 7), for some studies this has been useful.

Since the initial report of this line in 1999, these RIP-Cre mice have been used successfully to delete genes in pancreatic β-cells and the hypothalamus (7–12, 14–28) (Table 1). These studies have yielded important new insights into genetic pathways controlling β-cell development and function as well as metabolism controlled by the hypothalamus. In many of these studies the inactivation of the gene under investigation was associated with glucose intolerance, diabetes, and disrupted islet architecture. In some cases, the disruption of only one allele of the floxed gene led to profound physiological consequences (10). In many of these studies mice of a variety of different genotypes were used. While some investigators used mice carrying two floxed alleles and mice harboring only the RIP-Cre transgene as controls (7, 12, 17, 19, 21, 27), others used only floxed mice as controls and did not examine the effect of the RIP-Cre transgene only (9–11, 13–16, 18, 20, 22–24, 26, 28). In some cases the control group appeared to be a pool of different genotypes, including floxed mice and RIP-Cre mice (25). In some cases it was not clear whether RIP-Cre mice were used as controls (8). Very commonly the genetic background of the animals under investigation was also not specified.

The RIP-Cre mice were originally established in a B6D2 F2 hybrid background and subsequently bred for 12 generations into a C57BL/6 background. At this point they were provided to the Jackson Laboratory where they have been maintained for the past six years. Since most floxed alleles are generated in ES cells derived from one or another of the many different 129 substrains that are known to exist, we surmise that most of the studies described involve at some point the intermixing of these two different strain backgrounds. Moreover, it is also likely that the percentage of each strain may change over time, depending on the breeding strategy that is used.

The genes inactivated using the RIP-Cre mice encode transcription factors (*Stat3* (14, 16), *Stat5*,[3] *Foxa2* (24), *HNF-1β* (15), *HNF-4α* (22), *COUP-TFII* (10), *Tfam* (9), *ARNT* (24)), receptors (*IGF1* (25) glucocorticoid (13), *somatostatin* (8), c-*met* (26), and *insulin* (19)), *IRS2* (7, 17, 18), the Friedrich ataxin protein *frataxin* (21), a voltage-gated Ca^{2+} channel *Ca$_v$1.2* (23), PPARγ (27), *PKCλ* (28), and the *Men1* tumor suppressor (12, 20) (for details, see Table 1). With the exception of the Men1 studies, all others analyzed glucose homeostasis, performed glucose tolerance tests, and in some cases evaluated insulin secretion. In general mice carrying two floxed alleles and the RIP-Cre transgene were born without any defects but developed impaired glucose tolerance,

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[3] J.-Y. Lee and L. Hennighausen, unpublished data.

which in many cases progressed to overt diabetes mellitus. Impaired glucose tolerance frequently correlated with impaired insulin secretion (8, 14, 15, 19, 21–26, 28). Interestingly, in some cases mice that carried one floxed allele and the RIP-Cre gene already developed glucose intolerance (10). Moreover, histological evaluations revealed in several cases abnormal islets structures with α-cells interspersed within the islets (14, 16).

In a recent study aimed to explore the physiological significance of the cytokine-induced transcription factors *Stat3* and *Stat5* in pancreatic β-cells, the laboratory of L. H. and J.-Y. L. inactivated the respective genes using RIP-Cre mice obtained from the Jackson Laboratory and Pdx1-Cre mice obtained from the Melton laboratory (29). Mice, which carried two floxed *Stat5* alleles and the RIP-Cre transgene, developed glucose intolerance as early as 6 weeks of age, and isolated islets from these mice failed to adequately secrete insulin. In contrast, deletion of the *Stat5* locus using Pdx1-Cre did not lead to glucose intolerance in 2–3 months old mice.[4] This result prompted us to analyze glucose tolerance in mice carrying only the RIP-Cre transgene. To our surprise the RIP-Cre mice developed glucose intolerance. Since this observation affected the interpretation of the importance of *Stat3* in β-cell physiology (14, 16), we also deleted the *Stat3* gene using a Pdx1-Cre transgene. A complete deletion was obtained using the latter transgene, but no glucose intolerance was observed (30).

RIP-Cre Mice Are Glucose Intolerant—Three laboratories (of L. H., M. F. W., and M. R.) have performed studies to explore glucose tolerance and insulin secretion in RIP-Cre mice. In the Hennighausen laboratory RIP-Cre mice were obtained from the Jackson Laboratory in a C57BL/6J background. They were originally bred with mice carrying floxed *Stat5* alleles in a 129×C57BL/6 background. Mice were fed an open formula National Institutes of Health-specified diet for maintenance, growth, and reproduction. To exclude the possibility that the physiology of the RIP-Cre mice had changed in our mouse facility in these strain backgrounds, Lee and Hennighausen again obtained RIP-Cre and control mice in a pure C57BL/6J background from the Jackson Laboratory. At 2 months of age the RIP-Cre mice already exhibited glucose intolerance, which was more profound in females (Fig. 1). Although there was no difference in fasting blood glucose levels, the glucose levels after intraperitoneal injection of glucose (2 g/kg body weight) was significantly higher than those of control (C57BL/6J) mice in all time points measured.

Similar findings were obtained in the laboratories of Morris White (Fig. 2) and Michael Ristow (Fig. 3). To assess the ability of the pancreatic β-cells to secrete insulin, Lin and White measured blood insulin levels in 14–16-h fasted RIP-Cre mice at 2, 5, 15, and 30 min after injection of glucose (3 g/kg body weight). As expected, wild type littermates displayed a 2.4–3.4-fold increase in circulating insulin 2 min after glucose injection; however, circulating insulin did not increase in male or female RIP-Cre mice until 30 min after the glucose injection (Fig. 2*A*). These results suggest that impaired glucose stimulated insulin secretion may explain the mild glucose intolerance observed in RIP-Cre mice and the eventual suppression of blood glucose 2 h after glucose injection (Fig. 2*B*). Therefore, the investigation of glucose stimulated insulin secretion can be confusing in the RIP-Cre mice.

Ristow and colleagues (21, 30) discovered that targeted deletion of the *frataxin* gene in pancreatic β-cells caused overt diabetes mellitus in mice. When comparing RIP-Cre mice with wild-type or *frataxin^{fl/fl}* littermates, significant, albeit less pronounced, differences in regards to glucose tolerance and insulin secretion were observed (Fig. 3). Specifi-
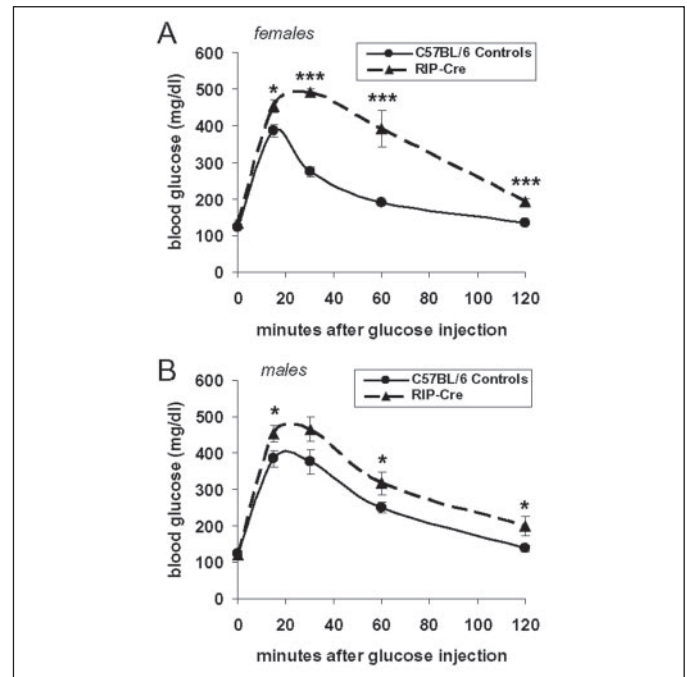
FIGURE 1. **Impaired glucose tolerance of RIP-Cre mice in a pure C57B/6 background.** Females (*A*) and males (*B*) were obtained from the Jackson Laboratory, and glucose tolerance was evaluated upon intraperitoneal injection of glucose (2 g/kg body weight). All results were reported as mean ± S.E. for the equivalent groups and compared with independent t tests (unpaired and two-tailed). Data are from the laboratory of Lothar Hennighausen. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.
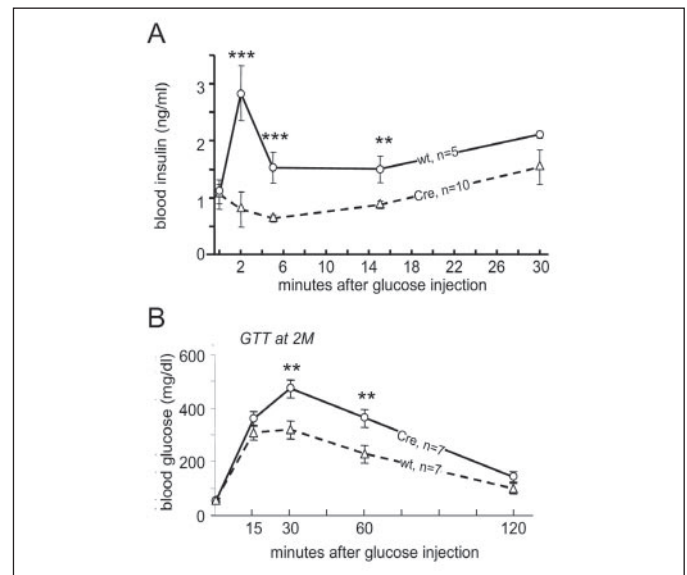

FIGURE 2. **Glucose-stimulated insulin secretion and glucose tolerance in wild type and RIP-Cre mice.** *A*, blood insulin levels before and after intraperitoneal injection of D-glucose (3 g/kg body weight). *B*, glucose tolerance test were performed after intraperitoneal loading with D-glucose (2 g/kg body weight) on 2-month-old mice. Results are mean values ± S.E. Data from the laboratory of Morris F. White. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

cally, impaired glucose tolerance after intraperitoneal injection was already observed in young mice at 6 weeks of age (Fig. 3*A*). This impairment of glucose tolerance persisted in adult mice at 18 weeks of age (Fig. 3*B*) and beyond (data not shown). Last, in older mice at 36 weeks of age a specific impairment of insulin secretion was observed suggesting a loss of the so-called first phase secretion 4 min after intraperitoneal injection of glucose (Fig. 3*C*).

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[4] L. Hennighausen and J.-Y. Lee, unpublished data.
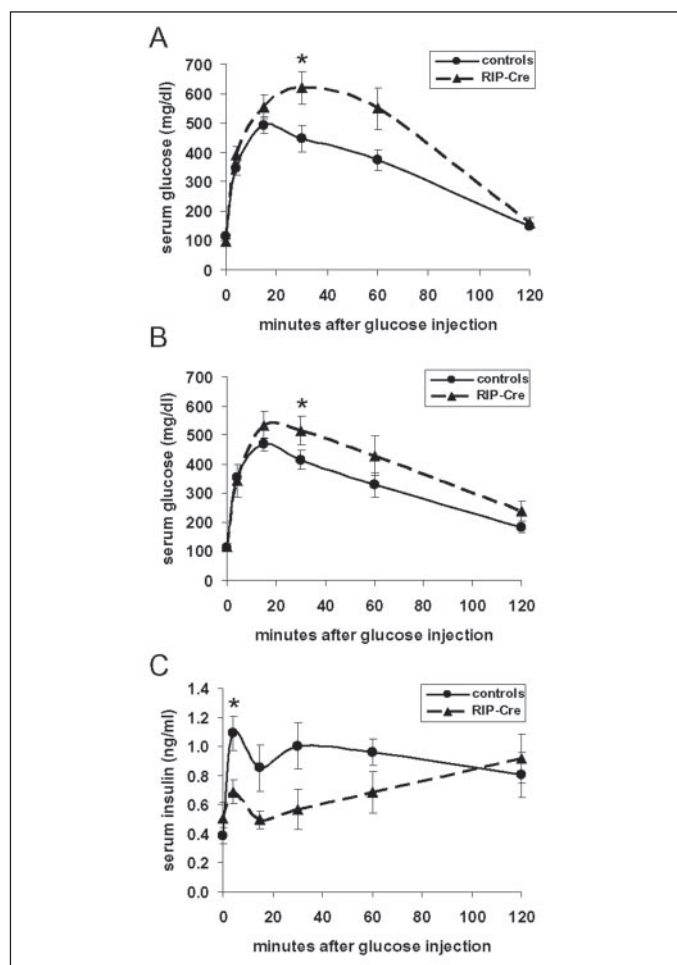
FIGURE 3. **Impaired first phase insulin secretion and impaired glucose tolerance of RIP-Cre mice in a background containing at least 95% C57B/6.** *A*, serum glucose values before and after an intraperitoneal glucose load in 6-week-old RIP-Cre mice compared with RIP-Cre-negative littermates. *B*, serum glucose values before and after an intraperitoneal glucose load in 18-week-old RIP-Cre mice compared with RIP-Cre-negative littermates. *C*, serum insulin values before and after an intraperitoneal glucose load in 36-week-old RIP-Cre mice compared with RIP-Cre-negative littermates. Data were from the laboratory of Michael Ristow. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Although there is now evidence from our laboratories that RIP-Cre mice by themselves can develop glucose intolerance and possibly impaired first-phase insulin secretion, other studies have used RIP-Cre mice as controls and did not observe glucose intolerance. However, many published studies have apparently not used RIP-Cre mice as controls. Could strain differences contribute to the observed glucose intolerance? The original strain of RIP-Cre mice was developed in a C57BL/6×DBA-2 hybrid background (5), and the question of whether these mice may have been glucose intolerant was not addressed.

Many of the publications that have made use of these mice have not specified the background of the mice used. However, since gene targeting is typically performed in ES cells derived from 129 strain mice, it is prudent to speculate that crosses involving floxed mice and RIP-Cre mice will contain a mixture of these two strains. Investigators often prefer using C57BL/6J mice, in part because of the small litter size and poor reproductive performance of 129 strain mice. Thus, in some studies mice carrying the floxed gene of interest were crossed into a C57BL/6 background and the resulting mice containing the Cre transgene were essentially C57BL/6 (see Table 1). Other studies stated the different strain backgrounds (including C57BL/6, CBA-J, 129, and DBA-2) but did not specify the percentage of each strain. Most studies did not go

into any details concerning the strain background. While approximately 15 investigators initially obtained the RIP-Cre mice from the original laboratory, since then most have obtained them from the Jackson Laboratory. Upon the generation of the RIP-Cre transgenic mice, the Magnuson laboratory bred the mice into a C57BL/6J background, and the mice shipped to investigators during this period contained different degrees of C57BL/6. One study described here (by the laboratory of L. H. and J.-Y. L.) did demonstrate that RIP-Cre mice displayed similar glucose intolerance in a pure C57BL/6 background and in a mixed 129 x C57BL/6 background. RIP-Cre mice used in the Ristow laboratory were obtained from R. N. Kulnarni in 2000 and were subsequently crossed with C57BL/6 (Charles River) until >92.5% C57BL/6. It is possible that strain differences might modulate the glucose intolerance observed in RIP-Cre mice. C57BL/6 mice are known to be highly prone to glucose intolerance and development of diabetes. In fact, this may not be a good strain for studying islet function, although it is commonly used nonetheless. Glucose intolerance in RIP-Cre mice could be explained, at least in part, by the observation that insulin secretion from isolated islets is impaired in these mice. It is also possible that Cre could cause recombination of pseudo loxP sites in β-cells and thereby compromise their function, although this has never been investigated. Pdx1-Cre and RIP-Cre mice efficiently delete genes in β-cells, but the exact levels of Cre recombinase in these strains are not known. However, in the original screen of RIP-Cre transgenic mice the line with the highest expression of Cre was selected, and it is possible that excessive levels of this prokaryotic protein adversely affect the physiology of pancreatic β-cells. Indeed, high levels of Cre have been reported to be toxic in some mammalian cells. Mouse embryonic fibroblasts and NIH 3T3 cells (1, 2, 4) and some human cell lines (4) can be sensitive to the continuous presence of Cre. Moreover, illegitimate chromosomal rearrangements have been detected in postmeiotic spermatids in transgenic mice expressing Cre (3). It is possible that such toxicity is the result of endogenous target sites for Cre in the mammalian genome (31). However, reports of toxicity have been rare, and in most cells levels of Cre obtained in transgenic and gene knock-in mice seem to be tolerated without recognizable physiological defects. One of our laboratories (L. H.) has extensively used transgenic mice expressing high levels of Cre recombinase in mammary stem cells and mammary epithelial cells at different stages of mammary development and never observed adverse effects. Integration of the RIP-Cre transgene in a locus relevant for the physiology of cells would be an alternative explanation for our findings.

CONCLUSIONS

Here we demonstrate that mice expressing the RIP-Cre transgene can develop glucose intolerance and impaired insulin secretion, even in the absence of genes targeted by loxP sites. The pathology is more severe in females than in males. This glucose intolerance has been observed in a pure C57BL/6 background and in mixed backgrounds containing 129 and C57BL/6. Although the β-cell dysfunction in RIP-Cre mice is not limited to a C57BL/6 background, it is possible that the degree of glucose intolerance is influenced by the strain background. Findings described in this paper potentially affect publications that have not used RIP-Cre mice as controls. The kinetics and magnitude of serum glucose levels in RIP-Cre mice upon glucose challenge is reminiscent of that observed in mice carrying the RIP-Cre and floxed genes from several studies. This tentatively suggests that some conclusions of the studies lacking appropriate controls are in question. The analysis of littermate controls carrying either the RIP-Cre transgene alone or in combination with one floxed allele will be necessary to identify potential problems. However, to avoid any problems it will be desirable to choose a mouse

strain in which Cre transgene expression in pancreatic β-cells does not result in glucose intolerance and diabetes. Toward this goal several approaches could be taken. These include the generation of mice in which the Cre transgene is inserted by homologous recombination into the endogenous loci of one of the two insulin genes. Moreover, such an approach could be combined with inducible components, either by fusing Cre with components of steroid receptors (32) or through the introduction of a tetracycline-responsive promoter component (33, 34).

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